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Antioxidant Inhibitors Potentiate the Cytotoxicity of Photodynamic Therapy

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Abstract

Photodynamic therapy (PDT) is an increasingly popular anticancer treatment that uses photosensitizer, light, and tissue oxygen to generate cytotoxic reactive oxygen species (ROS) within illuminated cells. Acting to counteract ROS-mediated damage are various cellular antioxidant pathways. In this study, we combined PDT with specific antioxidant inhibitors to potentiate PDT cytotoxicity in MCF-7 cancer cells. We used disulphonated aluminium phthalocyanine photosensitizer plus various combinations of the antioxidant inhibitors: diethyl-dithiocarbamate (DDC, a Cu/Zn-SOD inhibitor), 2-Methoxyestradiol (2-ME, a Mn-SOD inhibitor), L-buthionine sulfoximine (BSO, a glutathione synthesis inhibitor) and 3-amino-1,2,4-Triazole (3-AT, a catalase inhibitor). BSO, singly or in combination with other antioxidant inhibitors, significantly potentiated PDT cytotoxicity, corresponding with increased ROS levels and apoptosis. The greatest potentiation of cell death over PDT alone was seen when cells were pre-incubated for 24 hours with 300 μ M BSO plus 10 mM 3-AT (1.62-fold potentiation) or 300 μ M BSO plus 1 μ M 2-ME (1.52-fold), or with a combination of all four inhibitors (300 μ M BSO, 10 mM 3-AT, 1 μ M 2-ME, 10 μ M DDC: 1.4-fold). Because many of these inhibitors have already been clinically tested, this work facilitates future *in vivo* studies.

Introduction

Compared with their normal counterparts, many types of cancer cells have increased intracellular levels of reactive oxygen species (ROS), reflecting a disruption of redox homeostasis (1, 2). The increase in ROS is attributed to intrinsic mechanisms (activation of oncogenes, aberrant metabolism, mitochondrial dysfunction, and loss of functional P53) and extrinsic mechanisms (inflammatory cytokines, an imbalance of nutrients and hypoxic environment), thought to either elevate ROS production or impair the ROS-scavenging capacity of tumour cells (1, 2, 3).

Cancer cells adapt to this persistent oxidative stress by developing an enhanced endogenous antioxidant capacity, the extent of which correlates with the aggressiveness of the tumour, while at the same time making the malignant cells resistant to anticancer strategies that rely on inducing ROS stress (2). Several therapeutic approaches to killing cancer cells involve elevating cellular ROS levels: photodynamic therapy (PDT), chemotherapy, radiotherapy, immunotherapy, hormone therapy and hyperthermia (3). These anticancer therapeutic approaches are only successful in causing cytotoxicity if the increase in ROS exceeds a threshold level that is incompatible with cellular survival (2). The effective final concentrations of ROS in cancer cells are thus pivotal for pro-oxidant cancer therapies and depend on the balance of the intrinsic ROS levels, the increase in ROS caused by the therapy, and the competing antioxidant capacity of the tumour cells (3).

Several mechanisms are thought to be involved in the protective cellular responses to PDT. These include: activation of redox sensitive transcription factors (causing an increase in detoxifying and antioxidant enzymes), activation of anti-apoptotic pathways, and over expression of heat shock proteins (inhibiting the formation of an active apoptosome), as

reviewed by Nowis *et al.* (4). Moreover, tumours upregulate antioxidant haeme oxygenase-1 (HO-1) and other cytoprotective molecules as an adaptive response against oxidative stress (5, 6). In addition, PDT treatment is antagonized by three major cellular antioxidant defence mechanisms: superoxide dismutase enzymes (Cu/Zn-SOD and Mn-SOD), the glutathione (GSH) system, and catalase (7, 8, 9, 10, 6).

Cellular antioxidant systems therefore represent a useful target to improve the therapeutic efficacy of ROS-mediated anticancer therapies. For instance, both radiotherapy (11, 12, 13) and platinum-based chemotherapy (14, 15) are augmented when combined with inhibitors of glutathione, or superoxide dismutases.

In this study, MCF-7 cancer cells were used to investigate whether combining PDT with inhibitors of the four main antioxidant defences: diethyl-dithiocarbamate (DDC, an inhibitor of Cu/Zn-SOD), 2-Methoxyestradiol (2-ME, an inhibitor of Mn-SOD), L-buthionine sulfoximine (BSO, an inhibitor of GSH synthesis) and 3-amino-1,2,4-Triazole (3-AT, an inhibitor of catalase), either singly or in combination, would augment PDT ROS-mediated cell death. In addition, we investigated whether: i) there was any correlation between the inhibition of specific antioxidant pathway(s) and sensitivity to PDT-induced death and ii) if there was any relationship between cellular ROS levels and cell death in the presence of the various antioxidant inhibitors. These approaches lead the way to the therapeutic use of antioxidant inhibition plus PDT to sustain a high intracellular level of ROS in cancer cells that would otherwise be resistant to oxidative stress, thereby improving existing PDT treatment and expanding its use to more aggressive tumour types.

Materials and Methods

The various experimental conditions and subsequent assays are summarised in Fig. 1

Cell cultures

Human breast adenocarcinoma cell line MCF-7 was a kind gift from Dr. Marilena Loizidou, UCL Medical School, London. MCF-7 cells were maintained as monolayers in 25 mM glucose DMEM supplemented with: 100 µU/ml streptomycin, 100 µU/ml penicillin and 10% heat-inactivated fetal calf serum (FCS). For experiments, cells were grown in triplicate at a density of 1×10^5 cells/well in 500 µl growth medium in 24-well tissue culture plates and allowed to attach for 24 hrs to attain ~100% confluence.

PDT treatment

All incubations and washes prior to PDT were carried out under subdued lighting. Thirty minutes prior to PDT, standard serum-containing DMEM was replaced with fresh medium without serum, containing 5 µg/ml AlPcS₂ (a gift of Prof David Phillips, Imperial College. Stock 5mg/ml in water (16)). Then cells were rinsed 3 times with warm PBS, followed by warm phenol red-free DMEM supplemented with 1% pen/strep and 10% FCS. Test samples were immediately exposed (for 15 mins) to 28.6 J/cm² water-filtered halogen white light from a 500W bulb (or not in the case of dark cytotoxicity). Samples were then incubated under standard cell culture conditions for a further 24 hrs post PDT in the dark, and then assayed for viability.

Cell viability analysis

Cells were washed three times with PBS and the collected culture medium and washes were combined to ensure that any detached cells were not lost. The remaining attached cells were removed with trypsin-EDTA and the cell suspension combined with the cells already collected and the total cell number was determined by haemocytometer. The cells were incubated with 20 µg/ml propidium iodide (PI) on ice and analysed by flow cytometry using

a FACSCaliburTM cytometer. For each sample, 10,000 events were acquired on a logarithmic scale and the gating of single cells was achieved by analysis of forward and side scatter dot plots using BD CellQuestTM Pro software. PI fluorescence intensity was measured in FL-3 with an emission wavelength of 670 nm. For measurement of apoptosis 24 hrs after PDT, cells were incubated with 1:100 Annexin V-FITC (Sigma, A9210) for 15 min at room temperature in the dark and then analysed by flow cytometry (as detailed above) but using FL-1 with an emission wavelength of 530 nm.

Measurement of ROS

ROS levels were determined by flow cytometry using the fluorescent probes 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) or dihydroethidium (DHE). Cells were seeded in 24-well tissue culture plates at a density of 2×10^5 cells/ml and incubated at 37 °C overnight and then treated with 5 µg/ml AlPcS₂ and the various inhibitors for the indicated times. After incubation, the photosensitizer-containing medium was removed and the cells rinsed 3 times with warm PBS. Fresh phenol red-free culture medium with 10 µM DCFH-DA or 10 µM DHE was added under subdued light conditions and the test samples then exposed to 28.6 J/cm² water-filtered white light (or not in the case of dark cytotoxicity). The cells were then washed twice with cold PBS, trypsinized and centrifuged for 5 minutes at 550 g and at 4 °C. The cell pellet was resuspended in 200 µl cold PBS and probe fluorescence was measured using FACSCaliburTM cytometer by collecting 10,000 events for each sample. ROS levels were expressed as mean fluorescence intensity (MFI) as calculated by BD CellQuestTM Pro software. ROS was also measured in a cell-free system in 96-well plates (Corning: black, clear-bottom, flat) comprising fresh phenol red-free culture medium containing 0.2 µM DCFH-DA, 5 µg/ml AlPcS₂, and the various inhibitors. Each plate was illuminated, as detailed above, (or maintained in the dark) and the DCF fluorescence was

measured immediately using a plate reader (BMG labtech, FLUOstar optima; *Ex* 485 nm, *Em* 520 nm, gain 300).

Results

Initially, we identified a range of doses for each antioxidant inhibitor, in the absence of photosensitizer, that did not cause significant MCF-7 cell death or morphological change during 24 hr, but were nevertheless within the accepted working range for inhibition (17, 18, 19, 20, 21). In half of these initial experiments, cells were illuminated 30 minutes after adding the antioxidant inhibitor in order to determine if any inhibitor had an intrinsic photosensitizing activity (Fig. 2 and Fig. S1). Neither 3-AT nor BSO were found to be toxic or phototoxic at any of the doses used (Fig. 2C,D). By contrast, 2-ME led to the dose-dependent appearance of many rounded and floating cells, both in the dark and in photo-irradiated samples (Fig. S1). However, cell viability analysis demonstrated this was not due to cell death (Fig. 2A), suggesting that 2-ME affected cell adhesion, as has been proposed before (22).

DDC demonstrated a concentration dependent increase in cell death that, at the highest dose (30 μ M), was more pronounced when samples were illuminated (Fig. 2B and Fig. S1), suggesting that DDC may have some innate photosensitizing activity and/or it interferes with the antioxidant systems that normally counteract ROS produced during light exposure by endogenous chromophores, such as riboflavin and porphyrin.

As a result of these dose-toxicity tests, we chose three concentrations of each inhibitor that were minimally toxic (in the absence or presence of light) for further analysis in combination with 5 μ g/ml AlPcS₂ photosensitizer. These concentrations were: 2-ME (0.3, 1 and 3 μ M), DDC (1, 3 and 10 μ M), 3-AT (1, 3 and 10 mM), and BSO (30, 100 and 300 μ M).

Dark and photo toxicity studies of single antioxidant inhibitors with photosensitizer.

MCF-7 cells were co-incubated with antioxidant inhibitor and AlPcS₂ photosensitizer for 30 min prior to PDT and then maintained in the dark with antioxidant inhibitor for a further 24hr prior to analysis of cell viability and cell number per well (Table 1: 0.5 hr and Fig. 3).

The combination of AlPcS₂ with the different concentrations of DDC, or 3-AT or BSO for 30 minutes in the dark demonstrated no altered cytotoxicity after 24 hr, although there was a non-significant trend towards fewer cells with increasing antioxidant inhibitor concentration (Table 1: dark 0.5 hr and Fig. 3B-D). Morphologically, there were very few rounded and floating cells even at the highest concentrations of these three antioxidant inhibitors (Fig. 3B-D). By contrast, the combination of AlPcS₂ and 2-ME in the dark showed a dose-dependent increase in the number of rounded floating cells (Fig. 3A), as had been seen in the 2-ME-only experiments (Fig. S1). However, unlike the 2-ME-only experiments, the combination of 2-ME and photosensitizer in the dark reduced the percentage cell viability in a dose dependent manner, with 3 μ M 2-ME achieving a small but highly significant ($P<0.001$) decrease in cell viability when compared to AlPcS₂ only (Table 1: dark 0.5 hr, cell viability).

Unlike the situation in the dark, the combination of AlPcS₂ with any of the antioxidant inhibitors for 30 minutes, followed by PDT, caused an increase in the number of floating cells (Fig. 3), a dose-dependent trend of decreasing total cell number (Table 1: light 0.5 hr, cell number) and, for 2-ME, a dose-dependent trend of decreasing cell viability (Table 1: light 0.5 hr, cell viability).

The numbers of viable cells per well in the dark and after PDT treatment were calculated from their respective percentage cell survival and total cell number per well (Table 1). Dividing the values for the total viable cells after PDT treatment by total viable cells in the dark, a viability ratio was obtained (Table 1: viability ratio). This ratio normalised any

differences due to antioxidant inhibitors alone and allowed any specific PDT potentiating effect to be determined.

Cells treated with AlPcS₂ alone or in combination with the different antioxidant inhibitors always produced a significant ($P<0.001$) reduction in viability ratio compared to the control samples without photosensitizer or inhibitor.

Importantly, however, three inhibitor treatment conditions significantly potentiated ($P<0.05$) the photo toxicity of AlPcS₂ following 30 minutes pre-incubation (Table 1: viability ratio 0.5 hr). These were 1 μ M 2-ME, 100 μ M and 300 μ M BSO. DDC and 3-AT both demonstrated a non-significant trend to potentiate photo toxicity at the highest concentrations used (10 μ M DDC or 10 mM 3-AT).

Optimising the pre-incubation time with single antioxidant inhibitors.

The short-term incubation (30 minutes) of cells with antioxidant inhibitors helped to establish a single concentration for each antioxidant inhibitor that was not significantly dark toxic, but produced a reduction in the viability ratio, compared to AlPcS₂ alone. These concentrations were 1 μ M 2-ME, 10 μ M DDC, 10 mM 3-AT and 300 μ M BSO and were chosen for studies of longer-term (1 hr and 24 hr) pre-incubation before PDT treatment. AlPcS₂ photosensitizer was pulse-loaded into cells 30 minutes prior to PDT and cells were then maintained in the dark for a further 24hr before assessing cell viability, cell number, and viability ratio (Table 1: 1 hr and 24 hr, and Fig. 4).

In dark toxicity studies, the combination of AlPcS₂ with any of the specified antioxidant inhibitors resulted in very few rounded floating cells and did not significantly affect the percentage cell viability compared to no-drug/no-photosensitizer control, after either 1 hr (Fig. 4A and Table 1: dark 1 hr, cell viability) or 24 hr (Fig. 4B and Table 1: dark 24 hr, cell

viability) pre-incubation. However, there was a non-significant trend towards fewer cells compared to AlPcS₂ alone after 1 hr pre-incubation (Table 1: dark 1 hr, cell number) or 24 hr pre-incubation (Table 1: dark 24 hr, cell number). For either pre-incubation time, the greatest decrease in total cell number per well in the dark was achieved using a combination of AlPcS₂ and 10 mM 3-AT (Table 1: dark).

Upon photo-illumination, the combination of AlPcS₂ with any of the antioxidant inhibitors led to an increase in floating cells in 1 hr (Fig. 4A) and 24 hr (Fig. 4B) pre-incubated samples, compared to corresponding dark controls.

The combination of AlPcS₂ with almost all of the specified antioxidant inhibitors showed a non-significant trend of decreased cell number and decreased percentage cell viability following PDT when compared to AlPcS₂-PDT alone, both in 1 hr and 24 hr (Table 1: light) pre-incubated samples. The exception was with 300 μ M BSO, which showed a significant ($P<0.05$) decrease in cell viability in 24 hr pre-incubated samples (Table 1: light 24 hr, cell viability).

For 1 hr and 24 hrs pre-incubated samples, each of the antioxidant inhibitors demonstrated a trend to potentiate AlPcS₂-PDT, (Table 1: viability ratio). However, it was only 300 μ M BSO after 24 hr pre-incubation that achieved a statistically significant reduction ($P<0.05$) in viability ratio compared to AlPcS₂ alone (Table 1: viability ratio 24 hr, and Fig. 5C left side graph).

Combinations of antioxidant inhibitors

We selected four combinations of inhibitors, motivated by the antioxidant systems they are known to target (Fig. 9): 1 μ M 2-ME plus 10 μ M DDC target Mn-SOD and Cu/Zn-SOD respectively, thereby inhibiting the breakdown of singlet oxygen to hydroperoxides. 10 mM 3-AT plus 300 μ M BSO target both catalase and glutathione synthesis, thereby preventing the

breakdown of hydroperoxides. 1 μ M 2-ME plus 300 μ M BSO target both Mn-SOD and glutathione synthesis (and this particular pairing was chosen since, as individual inhibitors they showed the greatest potentiation of cytotoxicity). Finally, a cocktail of all 4 inhibitors was used to target all the major antioxidant systems. Each combination of inhibitors was added to cells 30 minutes, or 1 hr, or 24 hrs before PDT and then analysed 24 hrs after PDT.

Dark toxicity studies showed that the combination of AlPcS₂ plus 30 minutes or 1 hr pre-incubation with the antioxidant inhibitor mixtures were not toxic to the cells in the dark (Table 2: dark 0.5 hr and 1 hr). By contrast, with 24 hrs pre-incubation, the combination of AlPcS₂ with either 10 mM 3-AT plus 300 μ M BSO, or the four inhibitor cocktail showed a small but significant decrease ($P<0.05$) in percentage cell viability compared to AlPcS₂ alone (Table 2: dark 24 hr, cell viability).

In phototoxicity studies, each of the different antioxidant inhibitor combinations, at every pre-incubation time, demonstrated a trend of decreased cell number, which in several cases was statistically significant (Table 2: light, cell number). For each inhibitor combination, longer pre-incubation times gave a greater decrease in cell number and decrease in cell viability (Table 2).

Similarly, for each antioxidant inhibitor combination, increases in pre-incubation time gave progressively significant decreases in viability ratios, compared to AlPcS₂ alone (Table 2: viability ratio, and Fig. 5 right side graphs). Thus, 30 min pre-incubation provided no statistically significant decrease in viability ratio. 1 hr pre-incubation yielded a significantly decreased ($P<0.05$) viability ratio for the inhibitor cocktail (1.27-fold decrease compared to AlPcS₂ alone) (Table 2: viability ratio, and Fig. 5B right side graph). Finally, in samples pre-incubated for 24 hrs, any of the inhibitor combinations significantly decreased the viability ratio compared to AlPcS₂ alone: by 1.35-fold for 1 μ M 2-ME plus 10 μ M DDC ($P<0.05$), by 1.62-fold for 10 mM 3-AT plus 300 μ M BSO ($P<0.001$), by 1.52-fold 1 μ M 2-ME plus 300

μM BSO ($P<0.01$), and by 1.4-fold for the cocktail ($P<0.01$) (Table 2: viability ratio, and Fig. 5C right side graph).

Understanding the mechanisms of antioxidant inhibitor potentiated PDT

I. Apoptosis

Next, it was assessed whether AlPcS₂ plus the antioxidant inhibitors, either singly or in combination, led to apoptosis as assessed by annexin V-FITC flow cytometry 24 hrs after PDT (or dark control).

In the dark, none of the treatment conditions significantly increased the proportion of annexin V-positive cells when compared to no photosensitizer control (Fig. 6 left side graphs).

All AlPcS₂-PDT treatments produced an increase in annexin V-positive cells over light-exposed no photosensitizer controls (Fig. 6 right side graphs). However, only two antioxidant inhibitor combinations significantly increased the proportion of annexin V-positive cells compared to AlPcS₂-PDT alone, and both of these occurred following 24 hr pre-incubation. They were: 10 mM 3-AT plus 300 μM BSO ($P<0.05$), and the inhibitor cocktail ($P<0.05$) (Fig. 6C right side graph). In future experiments, it will be interesting to examine both earlier and later patterns of apoptosis in order to better understand the mechanisms and kinetics of cell death with each of the antioxidant inhibitors.

II. Analysis of ROS levels

At the end of each pre-incubation period, the intracellular ROS levels were assayed during illumination (or in the dark) using dichlorofluorescein (DCF) to detect general ROS (Fig. 7), or dihydroethidium to detect superoxide anions (Fig. 8). For each type of analysis, ROS values were expressed relative to the light treated AlPcS₂ sample of that pre-incubation time.

In the dark, none of the antioxidant inhibitors, singly or in combination, significantly increased ROS (Fig. 7 left side graphs) or superoxide levels (Fig. 8 left side graphs) compared to AlPcS₂ alone.

During illumination, the presence of BSO either singly (Fig. 7C right side graph) or in combination with 3-AT or 2-ME, but not the cocktail (Fig. 7A,C right side graphs), produced higher levels of ROS (using DCF) compared to the other inhibitors analysed. Conversely, the presence of 2-ME, or especially DDC, resulted in photo-induced ROS levels that were often lower than in samples treated with AlPcS₂ alone (Fig. 7 right side graphs). This was unexpected since the SOD inhibitors 2-ME or DDC would be predicted to raise intracellular ROS, notably superoxides.

Although DCF is commonly used as a general indicator of ROS, and is thought to reflect the overall oxidative status of the cell (23, 24), some studies have suggested that it is relatively insensitive to superoxides and hence not the appropriate probe for detecting superoxide radicals, as reviewed by Gomes *et al.* (25). To address whether this limitation might explain the apparent reduction in ROS observed with the two SOD inhibitors, dihydroethidium (DHE), a fluorescent probe that has relative specificity for superoxide anion radicals (O₂^{•-}) (25) was used (Fig. 8).

The combination of AlPcS₂ and 1 μM 2-ME increased the photo-induced O₂^{•-} levels in all the pre-incubation times (Fig. 8 right side graphs), with the maximum increase of 1.41-fold above AlPcS₂ alone after 24 hrs pre-incubation (Fig. 8C right side graph). Surprisingly, 10 μM DDC did not demonstrate any increase in O₂^{•-} levels (Fig. 8).

In cell-free media, 3-AT (alone or in combination with other inhibitors) resulted in a light-dependent increase in ROS, as measured by DCF fluorescence (Fig. S2). There are many differences between the cellular and cell-free systems, making direct comparisons

inappropriate. Nevertheless, this observation does imply that our DCF fluorescence results with 3-AT should be treated with caution.

Discussion

The success of PDT as an anti-tumour treatment is determined by the balance between photo-oxidative damage to cells by ROS (26), versus elimination of ROS by the scavenging activity of the cellular antioxidant systems (2, 27). In addition, there is increasing evidence that tumour cells initiate rescue mechanisms following PDT damage that include up-regulation of antioxidant systems (4, 6, 8, 28, 29). In this study, we demonstrate potentiation of ALPcS₂ PDT in MCF-7 cancer cells by inhibiting cellular antioxidant defences. This was achieved at antioxidant inhibitor concentrations that did not significantly increase cytotoxicity by themselves, making this work of interest for future pre-clinical studies.

The main cellular antioxidant defences that act against PDT are summarised in Fig. 9 and can be divided into two pathways. Initially, short-lived superoxides are converted to hydroperoxides by the superoxide dismutases, Cu/Zn-SOD and Mn-SOD. Subsequently, these hydroperoxides are broken down by glutathione and catalase.

Previous published results, using different cell lines, showed improved PDT cytotoxicity following single inhibition of glutathione (30), or catalase (31, 32), or Mn-SOD (8), or Cu/Zn-SOD (32), or HO-1 (33) which can itself upregulate SOD and catalase (34). However, unlike these previous studies, which had focussed on the effect of one or two antioxidants on PDT, our study directly compared inhibition of all these ROS scavenging systems, and then took this one step further by examining combinations of inhibitors.

Thus, using single antioxidant inhibitors at their optimum concentrations, we found that, in terms of protecting MCF-7 cancer cells against PDT: glutathione > Mn-SOD > catalase >

Cu/Zn-SOD (Table 3), consistent with data demonstrating that tumour cells up-regulate Mn-SOD(8) and glutathione (35, 36) following PDT induced damage.

We summarise our data for the 24hr pre-incubation period in Table 3, ranking antioxidants from most effective to least effective, in terms of augmentation of AIPcS₂-PDT cytotoxicity. Decreased viability ratio, increased ROS and increased annexin V staining all rank in the same order for the first three inhibitor combinations (BSO plus 3-AT, BSO plus 2-ME, and cocktail). This not only suggests a causal relationship between increased ROS levels and cell death, but indicates that hydroperoxide degradation, normally occurring jointly via catalase (inhibited by 3-AT) and glutathione (inhibited by BSO), is of greater importance in protecting MCF-7 cells against AIPcS₂-PDT than superoxide degradation, occurring jointly via Cu/Zn-SOD and Mn-SOD.

However, a disparity occurs between cell kill and ROS levels for some single inhibitors. For instance (in Table 3), BSO alone gives the 3rd highest ROS increase, but only the 5th highest PDT-specific cell kill. Conversely, inhibition of catalase with 3-AT gives the 5th highest ROS increase but only the 7th highest PDT-specific cell kill. In cell-free experiments, 3-AT caused a light-dependent increase in DCF fluorescence. If this occurred via a non-ROS-mediated photochemical reaction then this could explain the disparity between apparent ROS levels and PDT cytotoxicity due to 3-AT. Alternatively, it is likely that a threshold level of ROS needs to be crossed before cytotoxic effects are obtained, as suggested by Trachootham et al (2). Thus, whilst we observed several significant increases in ROS, these may be below a level that is sufficient to cause cell death. In addition, the disruption of the redox balance by depletion of one antioxidant enzyme often results in compensatory changes in other enzyme activities, as well as in low-molecular weight antioxidants (37, 38).

The assessment of ROS production using DCF demonstrated that BSO, either singly or in combination with other inhibitors, produced the largest increase in ROS levels compared to

the other inhibitors. Glutathione is the major ROS-scavenging system in all cells (2) and its inhibition by BSO has previously been shown to be followed by an increase in ROS levels(39).

2-ME and 3-AT have previously been shown to increase the ROS levels in different cell lines (40, 41) and our results demonstrated a slight increase in ROS levels in the presence of 3-AT (using DCF) and 2-ME (using DHE) when compared to AlPcS₂ alone. DDC, on the other hand, consistently yielded reduced ROS levels compared to AlPcS₂ alone with both ROS assays and this agreed with results obtained by Han *et al.* (20) and Kimoto-Kinoshita *et al.* (42) who also observed a decrease in ROS in the presence of DDC. DDC is known to have both antioxidant and pro-oxidant effects in different cell systems (42, 43). As an antioxidant, it can act directly by inhibiting superoxide production or by blocking oxidoreductase enzymes such as xanthine oxidase that are involved in free radical production (43, 44) and this might explain the decreased ROS levels in the presence of DDC, either singly or in combination with other inhibitors. It may also explain why the cocktail of inhibitors only showed a slight increase in ROS, compared with other inhibitor combinations that did not include DDC.

In summary, the pre-treatment of MCF-7 cancer cells with antioxidant inhibitors prior to PDT (especially inhibitors of hydroperoxide degradation), causes ROS accumulation in the cells and enhances PDT cytotoxicity. It will be interesting to determine whether the elevated antioxidant capacity of many types of cancer cell results in a cancer-selective cell kill, compared to normal cells, when using antioxidant inhibitors together with PDT.

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Supporting material

Figure S1. Representative phase-contrast micrographs of MCF-7 cells in dark and photo toxicity studies, without ALPcS₂ photosensitizer. (See “figures” for legend).

Figure S2. The relative ROS levels in cell-free medium, determined by DCF fluorescence with various antioxidant inhibitors. (See “figures” for legend).

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Table 1. Individual inhibitors. The dark and photo toxicity effects on MCF-7 cells of AlPcS₂ with 2-ME, or DDC, or 3-AT, or BSO on percentage cell viability, cell number, and viability ratio after 0.5, 1 or 24 hrs pre-incubation with antioxidant inhibitors. Cell viability was assessed by PI exclusion assay. For each treatment condition, results represent the mean of four independent experiments for 0.5 hr and 24 hrs, and five independent experiments for 1hr (mean±SEM). Within each time-point, data were analysed by one way ANOVA with Tukey's multiple comparison test. The emboldened values showed statistically significant decreases compared to AlPcS₂ alone; *= *P*<0.05, ***= *P*<0.001.

Inhibitor concentration and pre-incubation time (hrs)	5 µg/ml AlPcS ₂		Dark		Light		Viability ratio
			Cell Viability (%)	Cell Number (10 ⁵)	Cell Viability (%)	Cell Number (10 ⁵)	
0	0.5	-	92.5±0.5	2.99±0.15	93.1±0.7	2.87±0.14	0.97±0.02
0	0.5	+	92.4±0.6	2.98±0.15	87.7±0.9	2.40±0.15	0.70±0.02
2-ME 0.3 µM	0.5	+	92.7±0.2	3.08±0.43	86.5±1.8	1.97±0.28	0.60±0.03
2-ME 1 µM	0.5	+	92.5±0.6	2.87±0.37	84.6±2.1	1.89±0.25	0.57±0.02*
2-ME 3 µM	0.5	+	88.8±0.2***	2.45±0.38	82.5±1.5	1.54±0.27	0.58±0.05
DDC 1 µM	0.5	+	91.6±1.1	2.72±0.17	88.9±0.6	2.01±0.12	0.72±0.06
DDC 3 µM	0.5	+	92.0±0.7	2.68±0.15	88.2±0.6	1.91±0.77	0.69±0.05
DDC 10 µM	0.5	+	91.6±0.8	2.60±0.14	88.4±1.4	1.79±0.08	0.64±0.03
3-AT 1 mM	0.5	+	93.1±1.2	2.77±0.11	87.7±1.7	1.85±0.12	0.63±0.05
3-AT 3 mM	0.5	+	93.7±1.2	2.72±0.06	88.3±1.7	1.85±0.16	0.64±0.05
3-AT 10 mM	0.5	+	94.1±0.7	2.65±0.11	88.9±1.7	1.70±0.11	0.59±0.04
BSO 30 µM	0.5	+	91.5±1.97	3.00±0.47	87.0±1.7	2.03±0.25	0.66±0.05
BSO 100 µM	0.5	+	92.4±1.64	3.06±0.43	85.8±2.2	1.84±0.26	0.56±0.01*
BSO 300 µM	0.5	+	93.6±1.22	2.99±0.49	86.3±2.0	1.79±0.28	0.57±0.03*
0	1	-	95.3±0.2	2.31±0.12	95.6±0.3	2.16±0.07	0.94±0.03
0	1	+	94.8±0.4	2.22±0.09	93.6±0.9	1.70±0.18	0.79±0.03
2-ME 1 µM	1	+	93.9±0.8	2.08±0.11	92.2±0.6	1.47±0.12	0.68±0.04
DDC 3 µM	1	+	94.2±0.5	2.05±0.13	92.5±0.3	1.44±0.11	0.67±0.04
3-AT 10 mM	1	+	93.7±0.8	1.76±0.21	91.2±1.2	1.36±0.19	0.72±0.04
BSO 300 µM	1	+	92.6±1.3	1.95±0.18	92.7±0.5	1.47±0.17	0.70±0.01
0	24	-	94.6±0.6	3.44±0.12	96.1±0.4	3.46±0.12	1.02±0.02
0	24	+	95.0±0.7	3.37±0.13	89.2±0.8	2.57±0.19	0.74±0.03
2-ME 1 µM	24	+	94.2±0.5	3.10±0.15	89.9±0.6	2.03±0.22	0.61±0.05
DDC 3 µM	24	+	94.8±0.8	3.17±0.16	89.9±1.0	2.25±0.19	0.67±0.03
3-AT 10 mM	24	+	93.3±0.7	2.96±0.15	87.4±0.8	2.04±0.14	0.65±0.05
BSO 300 µM	24	+	95.3±0.4	3.37±0.15	85.5±0.8*	2.10±0.20	0.55±0.03*

Table 2. Combinations of inhibitors. The dark and photo toxicity effects of AlPcS₂ with combinations of inhibitors on the viability and cell number after 24 hrs pre-incubation. MCF-7 cells were treated with AlPcS₂ in the presence of the indicated concentrations of antioxidant inhibitors for up to 24 hrs. Cell viability was assessed by PI exclusion assay. Results represent the mean of four independent experiments (mean±SEM) for each treatment condition and were analysed by one way ANOVA with Tukey's multiple comparison test. The emboldened values showed statistically significant differences compared to AlPcS₂ alone; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Inhibitors and pre-incubation time (hrs)	5 µg/ml AlPcS ₂		Dark		Light		Viability ratio
			Cell Viability (%)	Cell Number (10 ⁵)	Cell Viability (%)	Cell Number (10 ⁵)	
0	0.5	-	93.7±0.5	2.09±0.08	95.0±0.6	2.08±0.05	0.98±0.01
0	0.5	+	94.0±0.7	2.02±0.05	91.0±1.1	1.64±0.06	0.72±0.02
1 µM 2-ME 3 µM DDC	0.5	+	93.4±0.6	2.04±0.07	90.0±1.0	1.46±0.09	0.69±0.03
10 mM 3-AT 300 µM BSO	0.5	+	93.5±0.5	2.00±0.08	91.0±0.8	1.40±0.08	0.68±0.03
1 µM 2-ME 300 µM BSO	0.5	+	94.0±0.5	2.03±0.07	90.2±0.5	1.37±0.11	0.65±0.04
All	0.5	+	93.6±0.5	2.00±0.07	91.1±0.4	1.43±0.08	0.70±0.04
0	1	-	93.7±1.5	2.16±0.04	89.2±4.7	2.16±0.06	0.96±0.03
0	1	+	93.3±1.4	2.11±0.05	88.8±2.2	1.90±0.06	0.81±0.02
1 µM 2-ME 3 µM DDC	1	+	91.2±2.3	2.06±0.07	86.7±4.1	1.53±0.10*	0.71±0.04
10 mM 3-AT 300 µM BSO	1	+	90.4±2.6	1.93±0.08	84.1±6.1	1.61±0.07	0.77±0.05
1 µM 2-ME 300 µM BSO	1	+	90.6±3.2	2.03±0.07	86.0±3.5	1.54±0.11*	0.72±0.04
All	1	+	90.6±4.2	2.0±0.09	82.1±7.9	1.41±0.04**	0.64±0.04*
0	24	-	95.5±0.2	3.39±0.32	96.5±0.4	3.24±0.24	1.01±0.02
0	24	+	95.9±0.4	3.32±0.28	88.9±1.1	2.58±0.20	0.73±0.02
1 µM 2-ME 3 µM DDC	24	+	94.5±0.1	3.09±0.17	86.8±2.4	1.80±0.12	0.54±0.05*
10 mM 3-AT 300 µM BSO	24	+	92.7±1.0*	3.06±0.03	78.6±5.2	1.57±0.21**	0.45±0.09***
1 µM 2-ME 300 µM BSO	24	+	95.0±0.5	2.89±0.17	80.3±2.8	1.63±0.11*	0.48±0.04**
All	24	+	92.9±0.6*	2.59±0.11	79.8±3.4	1.57±0.07**	0.52±0.03**

Table 3. Summary of experimental results with 24 hrs pre-incubation. Antioxidant inhibitors are ranked by the greatest decrease in PDT-specific cell kill (Δ viability ratio), and then by greatest increase in ROS (Δ ROS), and by greatest increase in apoptotic cells (Δ annexin V). Each value represents the difference compared to the corresponding light-treated AlPcS₂ only control. Where this difference was statistically significant, it is indicated by asterisks; *= $P<0.05$, **= $P<0.01$, ***= $P<0.001$.

inhibitor(s)	antioxidant(s) inhibited	Δ viability ratio	Δ ROS	Δ annexin V
3-AT 10mM BSO 300 μ M	Catalase, glutathione	0.28 ***	2.79 **	15.37 *
2-ME 1 μ M BSO 300 μ M	Mn-SOD, glutathione	0.25 **	2.11 **	13.53
Cocktail 2-ME 1 μ M DDC 3 μ M 3-AT 10 mM BSO 300 μ M	Cu/Zn-SOD, Mn-SOD, catalase, glutathione	0.21 **	0.38	13.53 *
2-ME 1 μ M DDC 3 μ M	Cu/Zn-SOD, Mn-SOD	0.19 *	-0.31	7.50
BSO 100 μ M	Glutathione	0.14 *	1.30 **	3.35
2-ME 1 μ M	Mn-SOD	0.13	-0.30 (1.41 DHE)	2.86
3-AT 10 mM	Catalase	0.11	0.18	4.50
DDC 10 μ M	Cu/Zn-SOD	0.06	-0.68	-1.42

Figures

Figure 1. Cartoon summary of the various experimental conditions. MCF-7 cells were pre-treated with antioxidant inhibitors, loaded with photosensitizer, washed and illuminated, and then assayed immediately for ROS levels or after 24 hrs for cell viability.

Figure 2. Cell viability in the presence of antioxidant inhibitors, but without AlPcS₂ photosensitizer. MCF-7 cells were treated with various concentrations of antioxidant inhibitors and exposed to 28.6 J/cm² white light (dashed line) or kept in the dark (continuous line) and the percentage cell survival determined by propidium iodide exclusion assay. All conditions demonstrate minimal dark- or photo-toxicity, except (B) DDC at the highest concentrations.

Figure 3. Representative phase-contrast micrographs of MCF-7 cells in dark and photo toxicity studies. The cells were treated with AlPcS₂ and the different concentrations of antioxidant inhibitors for 30 min and then exposed to light or kept in the dark. The samples were incubated for a further 24 hrs under standard cell culture conditions in the presence of inhibitors, and the phase contrast micrographs acquired at the end of the incubation period.

Figure 4. Optimizing the pre-incubation time with antioxidant inhibitors. MCF-7 cells were treated with the specified concentrations of antioxidant inhibitors for A) 1 hr or B) 24 hrs, loaded with AlPcS₂ photosensitizer for 30 min, and then illuminated (or kept in the dark for the dark toxicity studies). The representative images were a snapshot of the center of the well 24 hr later, before analysis of the percentage cell viability.

Figure 5. The effect of pre-incubation time with antioxidant inhibitor(s) on the viability ratio of MCF-7 cells. Viability ratios were calculated from cell survival and cell number data from the dark and phototoxicity studies after A) 0.5 hr, B) 1 hr or C) 24 hrs pre-incubation with single antioxidant inhibitors (left side) or mixtures (right side). Results represent the mean of at least three independent experiments for each treatment condition (mean \pm SEM) and were analysed by one way ANOVA with Tukey's multiple comparison test. Statistically significant differences compared to the relevant AlPcS₂-only control (second bar in each graph pair) are indicated by asterisks; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Line (ratio of 1) indicates no difference in cell viability between the dark and photo toxicity.

Figure 6. The percentage of apoptotic MCF-7 cells, as determined by annexin V-FITC staining, following various pre-incubation times with antioxidant inhibitors, either maintained in the dark (left side) or after PDT (right side). MCF-7 cells were pre-incubated for A) 0.5 hr, B) 1 hr or C) 24 hrs with the specified antioxidant inhibitors and analysed 24 hr later. Results represent the mean of at least three independent experiments for each treatment condition (mean \pm SEM) and were analysed by one way ANOVA with Tukey's multiple comparison test. Statistically significant differences compared to the relevant AlPcS₂-only control (second bar in each graph pair) are indicated by asterisks; * = $P < 0.05$.

Figure 7. The relative ROS levels in MCF-7 cells, determined by DCF fluorescence, as a function of pre-incubation time with various antioxidant inhibitors. For each pre-incubation time the ROS levels are reported as a ratio, obtained by dividing the mean of each sample DCF fluorescence by the mean DCF fluorescence of the light treated AlPcS₂ sample (second bar in each light-treated graph, right side). Results represent the mean of three independent experiments for each condition (mean \pm SEM), analysed by one way ANOVA with Dunnett's

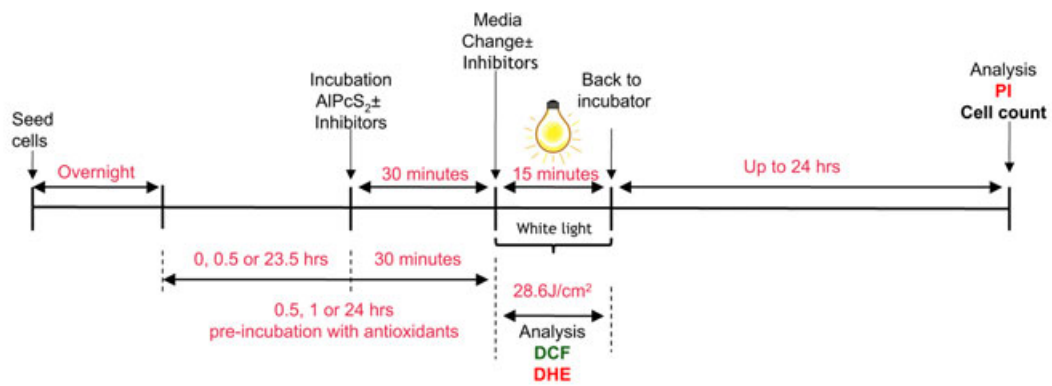
test. Statistically significant differences compared to the relevant AlPcS₂-only control (second bar in each graph pair) are indicated by asterisks; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Dotted line (ratio of 1) indicates no difference in DCF fluorescence compared to light-treated AlPcS₂-only samples.

Figure 8. The relative superoxide levels in MCF-7 cells, determined by DHE fluorescence, as a function of pre-incubation time with various antioxidant inhibitors. For each pre-incubation time the ROS levels are reported as a ratio, obtained by dividing the mean of each sample DHE fluorescence by the mean DHE fluorescence of the light treated AlPcS₂ sample (second bar in each light-treated graph, right side). Results represent the mean of three independent experiments for each condition (mean \pm SEM), analysed by one way ANOVA compared to the relevant AlPcS₂-only control (second bar in each graph pair). Dotted line (ratio of 1) indicates no difference in DHE fluorescence compared to light-treated AlPcS₂-only samples.

Figure 9. Summary diagram of the main cellular antioxidant systems responsible for detoxifying PDT-produced superoxides (O₂^{•-}) and the inhibitors that affect them.

Figure S1. Representative phase-contrast micrographs of MCF-7 cells in dark and photo toxicity studies, without AlPcS₂ photosensitizer. The cells were treated with the different concentrations of antioxidant inhibitors for 30 min and then exposed to light or kept in the dark. The samples were incubated for a further 24 hrs under standard cell culture conditions in the presence of inhibitors, and the phase contrast micrographs acquired at the end of the incubation period.

Figure S2. The relative ROS levels in cell-free medium, determined by DCF fluorescence with various antioxidant inhibitors. ROS levels are reported as a ratio, obtained by dividing the mean of each sample DCF fluorescence by the mean DCF fluorescence of the light treated AlPcS₂ sample (second bar in each light-treated graph, right side). Results represent the mean of three independent experiments for each condition (mean±SEM), analysed by one way ANOVA with Dunnett's test. Statistically significant differences compared to the relevant AlPcS₂-only control (second bar in each graph pair) are indicated by asterisks; **= $P<0.01$.



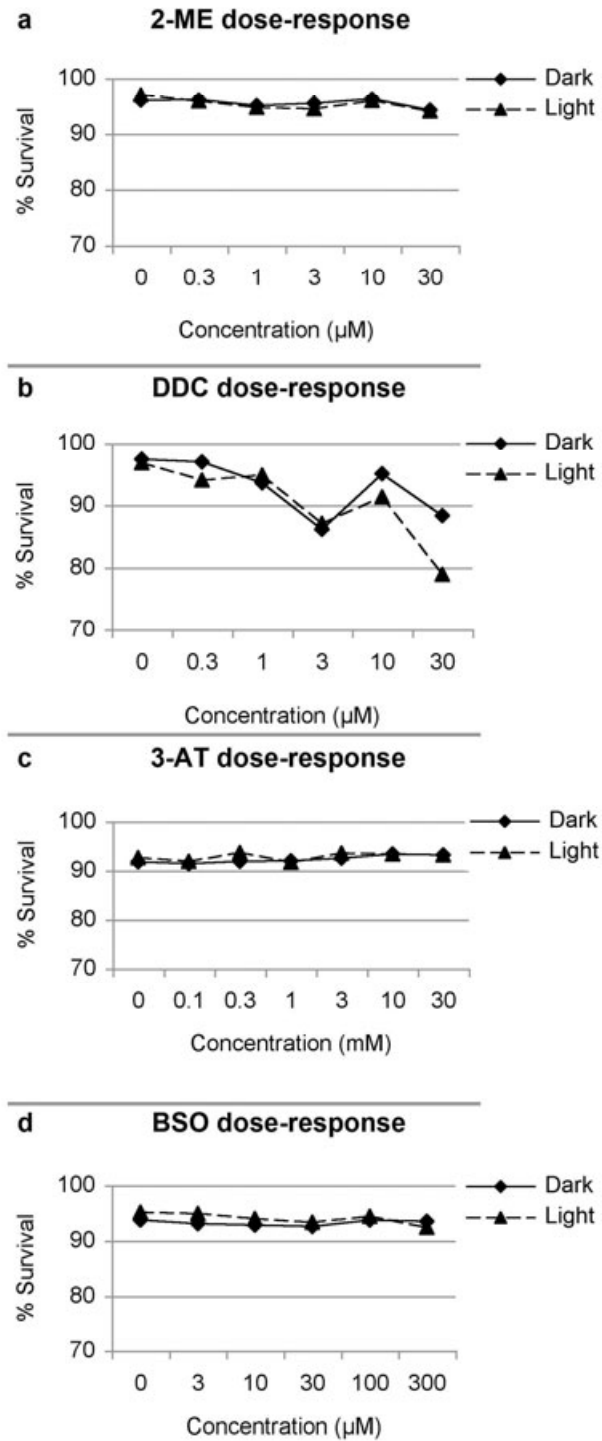


Figure 2

